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(54) Title: ANTIBODY DEPENDENT ENHANCEMENT (ADE) OF ALPHAVIRUS INFECTION

(57) Abstract: The present invention provides compositions and methods for delivering a nucleotide sequence to a cell using an alphavirus vector that is complexed with an enhancing antibody that specifically binds to the alphavirus vector. Venezuelan Equine Encephalitis vectors are preferred. The cell may be a cell *in vitro* or *in vivo*. Alternatively, the cell may be removed from a subject, administered the alphavirus vector *ex vivo* and then administered to a subject. Antigen-presenting cells are preferred, with dendritic cells being more preferred. Also provided are methods of producing an immune response in a subject, *e.g.*, for producing an immune response against an antigen associated with a pathogen or for immunotherapy of cancer of tumors.

Antibody Dependent Enhancement (ADE) of Alphavirus Infection

Related Application Information

This application claims the benefit of United States Provisional Application No. 60/151,718, filed August 31, 2000, and United States Provisional Application No. 60/177,435, filed January 21, 2000, which are incorporated by reference herein in their entirety.

Statement of Federal Support

The present invention was made with government support under grant number DAMD17-94-J-4430 from the United States Army Research and Development Command, and grant number F32-A109778 from the National Institute of Health. The United States government has certain rights to this invention.

Field of the Invention

The present invention relates to methods and compositions for delivery of nucleic acids, more particularly, alphavirus vectors and methods of administering the same.

Background of the Invention

The Alphavirus genus includes a variety of viruses, all of which are members of the Togaviridae family. The alphavirus genome is a single-stranded, messenger-sense RNA, modified at the 5′-end with a methylated cap, and at the 3′-end with a variable-length poly (A) tract. The viral genome is divided into two regions: the first encodes the nonstructural or replicase proteins (nsP1-nsP4) and the second encodes the viral structural proteins (Strauss and Strauss, (1994) *Microbiological Rev.* **58**:491-562, 494). Structural subunits consisting of a single viral protein, C, associate with

"dengue shock syndrome" that is associated with infection by dengue viruses (Morens, (1994) *Clin. Infectious Diseases* **19**:500). More recently, it has been proposed that ADE is involved in the pathogenesis of human immunodeficiency virus (HIV) and feline infectious peritonitis virus (FIPV) (*see, e.g.*, Füst, (1997) *Parasitology* **115** (Suppl): S127; Olsen, (1993) *Veterinary Microbiology* **36**:1).

ADE has been reported in cultured cells in connection with a wide variety of viruses, including flaviviruses, such as Dengue virus, West Nile virus, Murray Valley encephalitis virus, and Yellow fever virus; alphaviruses, such as tick-borne encephalitis virus, Semliki Forest virus, Western equine encephalitis virus, and Sindbis virus; lactate dehydrogenase virus; human respiratory syncytial virus; influenza A virus; rabies virus; feline infectious peritonitis virus (FIPV), human- and feline- immunodeficiency virus (HIV, FIV), and murine cytomegalovirus. (Olsen, (1993) *Veterinary Microbiology* **36**:1; Peiris et al., (1981) *J. Gen. Virol.* **57**:119).

Dendritic cells (DC) are postulated to play important roles in antigen presentation and initiation of several T cell dependent immune responses. DC have been demonstrated to be more potent antigen-presenting cells (APC) than are macrophages or monocytes. Moreover, it has been reported that DC stimulate T cell proliferation up to ten-fold more efficiently than do monocytes (Guyre et al., (1997) *Cancer Immunol. Immunother.* **45**:146, 147 col. 2). Accordingly, it would be desirable to target antigens or other therapeutic molecules to DC to produce an enhanced immune response, in particular, to improve the efficacy of vaccines and immunotherapeutic regimes.

Summary of the Invention

The present invention is based, in part, on the discovery that the efficacy of alphavirus vectors (*e.g.*, VEE vectors) may be enhanced (*e.g.*, improved, increased, and the like) in the presence of antibodies directed against the alphavirus. In particular, the infectivity of particular cells by alphavirus vectors may be enhanced in the presence of antibodies against the

Fig. 4 demonstrates that FcγR/II/III blocks ADE in BM-NS cells. VRP pre-incubated in VEE-specific antibodies were mixed with BM-NS cells that were pre-treated with either diluent, an isotype matched negative control Mab, anti-CR1 Mab or anti-FcγRII/III Mab. ADE was quantitated by FACS analysis of GFP-positive cells.

Fig. 5 presents data evaluating cell-surface expression of FcγRII/III. FACS analysis of FcγRII/III cell surface expression on BHK cells, on bone marrow cells grown either in GM-CSF alone (BM), GM-CSF and IL-4 (BM-IL-4) or GM-CSF and NS46 conditioned medium (BM-NS), two Langerhans cell lines, XS106 and XS 52, the macrophage cell line, RAW 264 and the B cell line, CH12.

Fig. 6 demonstrates that FcγRII/III positive cells of myeloid lineages differ in susceptibility to ADE mediated VEE infection. Three different bone marrow preparations grown either in GM-CSF alone, GM-CSF and NS 46 conditioned media or GM-CSF and IL-4, two Langerhans cell lines, XS106 and XS52, a macrophage cell line, RAW 264, a mature B cell line CH12LX, and Baby Hamster Kidney cells (BHK) were infected with GFP-VRP-3000 at an MOI of 1.0 either alone or pre-treated with control or VEE-specific antibodies. ADE was quantitated by FACS analysis of GFP-positive cell numbers.

Fig. 7 presents photomicrographs demonstrating that ADE alters VRP cell targeting *in vivo* towards dendritic-like cells. Lymph node sections from mice twenty-four hours post-inoculation with GFP-VRP-3014 **(A)** alone or pre-incubated in either **(B)** normal rabbit serum, **(C)** rabbit anti-VEE antibodies, or **(D)** VRP immune serum.

Fig. 8 provides photomicrographs demonstrating that VRP cell targeting is altered in VRP immune mice. Lymph node sections from mice twenty-four hours after either one dose of **(A)** 1×10^3 IU of GFP-VRP-3014,

shock syndrome or Dengue hemorrhagic fever and feline infectious peritonitis virus) that are marked by rapid elevation of viral titers and accelerated pathogenesis (Morens et al., (1994) *Clin. Infectious Diseases* **19**:500; Olsen, (1993) *Veterinary Microbiology* **36**:1). It is hypothesized that low concentrations of neutralizing antibodies complex with the virus, and the complex interacts with Fc receptors and/or complement receptors on the macrophage/monocytes. Binding of the alphavirus-antibody complex to cellular Fc receptors increases the concentration of virus on the cell surface, which results in enhanced infection of the cells.

Flynn et al., (1988) *Virology* **166**:82, and Linn et al., (1996) *J. Gen. Virology* **77**:407, have observed ADE of Sindbis and Ross River Virus infectivity, respectively, in cultured monocytes. However, these studies did not describe increased infectivity of dendritic cells (DC) in the presence of antibody. Moreover, these investigators did not report improved immunogenicity of alphavirus vaccines *in vivo* in the presence of antibody directed against the alphavirus.

Chanas et al., (1982) *J. Gen. Virol.* **58**:37, reported enhanced infectivity by Sindbis virus of macrophage-like cells by two neutralizing monoclonal antibodies at sub-neutralizing titers. When virus was administered intracerebrally to newborn mice in conjunction with various dilutions of antibody, neutralization (rather than enhancement) of viral-induced mortality was observed.

In contrast, the present investigations have found antibody-dependent enhancement of infectivity of APC (in particular, dendritic cells) by alphavirus vectors both *in vitro* and *in vivo*. It appears that alphavirus vectors may be advantageously targeted or directed toward APC in the presence of anti-alphavirus antibodies. Moreover, there may be an enhanced immune response elicited toward heterologous antigens encoded by the alphavirus vector in the presence of the enhancing antibody. ADE of alphavirus infectivity does not appear to be associated with any significant pathology, *i.e.*, does not have substantial adverse effects (*e.g.*, disease, morbidity or mortality in a subject administered the alphavirus vector in the presence of the

contacting the cell with an alphavirus vector carrying a heterologous nucleotide sequence in the presence of an antibody that enhances the infectivity of the vector. Preferably, the cell is contacted with an alphavirus vector comprising a heterologous nucleotide sequence and an antibody that specifically binds to the alphavirus vector, so that the heterologous nucleotide sequence is introduced into and, preferably, expressed in the cell.

By "contacting" a cell with an alphavirus vector and an antibody that specifically binds to the alphavirus vector, as used herein, it is intended that the cell is contacted with both the alphavirus vector and the antibody. Typically, both the alphavirus vector and the antibody vector are bound to the cell as a result of contacting the cell. While not wishing to be held to any particular theory of the invention, it is thought that both the alphavirus vector and the antibody will, at least transiently, be bound to the cell at the same time, although not necessarily to each other. Alternatively, the antibody may bind to the cell first and induce a change in the cell that enhances the efficacy of the alphavirus vector. The alphavirus vector may bind to the cell first, and the antibody may bind to the cell subsequently, or vice versa. Alternatively, the alphavirus vector and antibody may bind to the cell essentially simultaneously. In particular embodiments, the alphavirus vector and antibody forms a complex, and the complex contacts and binds to the cell, as described below. Alternatively, a complex may be formed between the alphavirus vector and the antibody after one or both have bound to the cell.

By "bind", "binding" and "bound", it is not necessarily intended that the alphavirus vector or the antibody directly bind to the cell. For example, the antibody may first bind to the cell (*e.g.*, at the Fc receptor) and the alphavirus vector may bind to the antibody to form a complex, and thereby be indirectly "bound" to the cell. Likewise, the alphavirus vector may first bind to the cell, and the antibody binds to the alphavirus vector that is already bound to the cell to form a complex therewith. As a further alternative, the alphavirus vector and antibody may bind to the cell at separate sites (*e.g.*, at their respective receptors), and may optionally bind to each other to form a complex.

The antibodies of the invention are directed against the alphavirus vector, preferably the virion of the alphavirus vector (*e.g.*, the antibody binds to the E1 glycoprotein and/or the E2 glycoprotein). In other words, according to this embodiment, the antibody recognizes an epitope(s) on the alphavirus, more preferably an epitope(s) in the alphavirus structural proteins, most preferably, in the alphavirus E1 glycoprotein and/or the alphavirus E2 glycoprotein. It is also preferred that the antibodies are anti-VEE antibodies.

It is further preferred that the antibody binds the alphavirus with high affinity, *e.g.*, with a dissociation constant of at least about 10^{-6}, preferably at least about 10^{-7}, more preferably at least about 10^{-8}, still more preferably at least about 10^{-9}. Alternatively stated, the antibody specifically binds to the alphavirus (as opposed to non-specific interactions). As used herein, the term "specifically binds to the alphavirus" is not intended to indicate that the antibody only binds to that particular alphavirus (*e.g.*, does not bind to other alphaviruses), although in particular embodiments, this may be the case.

Those skilled in the art will appreciate that the antibody may be neutralizing for alphavirus infection at sufficiently high concentrations. In addition, the antibody may by neutralizing in some cell types (*e.g.*, BHK cells) even at concentrations at which ADE is observed in other cells types (*e.g.*, DC). This phenomenon is contrary to most observations of ADE in the art, wherein ADE is typically believed to occur at sub-neutralizing titers of a neutralizing antibody (*see*, *e.g.*, Hawkes et al., (1967) *Virology* **33**:250). In contrast, the present investigations have found ADE of alphavirus infectivity of cells (*e.g.*, DC) at concentrations of alphavirus that are neutralizing in other cell types (*e.g.*, BHK cells).

The antibody is preferably present in an "enhancing" amount that is sufficient to increase or augment infectivity of the alphavirus vector in a cell as compared with the levels observed in the absence of the enhancing antibody. It is also preferred that the antibody is present in an "enhancing" amount that increases or augments an immune response to a heterologous antigen encoded by the vector as compared with the levels observed in the absence of the enhancing antibody, but insufficient to neutralize the alphavirus vector

present invention include, for example, Fab, F(ab')2, and Fc fragments, and the corresponding fragments obtained from antibodies other than IgG. Such fragments can be produced by known techniques.

Polyclonal antibodies used to carry out the present invention may be produced by immunizing a suitable animal (e.g., rabbit, goat, etc.) with the particular alphavirus (or alphavirus virion or glycoprotein), collecting immune serum from the animal, and separating the polyclonal antibodies from the immune serum, in accordance with known procedures.

Monoclonal antibodies used to carry out the present invention may be produced in a hybridoma cell line according to the technique of Kohler and Milstein, *Nature* **265**, 495-97 (1975). For example, a solution containing the appropriate antigen may be injected into a mouse and, after a sufficient time, the mouse sacrificed and spleen cells obtained. The spleen cells are then immortalized by fusing them with myeloma cells or with lymphoma cells, typically in the presence of polyethylene glycol, to produce hybridoma cells. The hybridoma cells are then grown in a suitable media and the supernatant screened for monoclonal antibodies having the desired specificity. Monoclonal Fab fragments may be produced in *Escherichia coli* by recombinant techniques known to those skilled in the art. *See, e.g.*, W. Huse, *Science* **246**, 1275-81 (1989).

Enhancing antibodies against alphaviruses may also be obtained by phage display techniques known in the art.

The antibody may be provided to the cell by any suitable method. For example, the antibody may be an exogenous antibody that is isolated or synthesized and provided to the cell. In particular preferred embodiments, the alphavirus vector and antibody may be combined into a formulation, and the cell is contacted with this formulation. Alternatively, the alphavirus vector is contacted with the cell prior to the antibody, or vice versa. As a further alternative, the cell may be concurrently contacted with the alphavirus vector and the antibody as separate formulations.

For *in vivo* applications, the alphavirus vector may be administered with an exogenous antibody of the invention. The antibody may be administered

In addition, exogenous antibody may advantageously be provided in those situations in which a more rapid and aggressive treatment is desired, *i.e.*, when it would be undesirable to wait for the subject to mount an immune response and produce anti-alphavirus antibodies.

The present invention may be practiced with any cell known in the art in which antibody-enhanced infectivity of the alphavirus is observed. Additionally, the present invention may be practiced with any cell in which antibody-mediated targeting of alphavirus vectors is observed. The present invention may further be practiced in any suitable cell in which an enhanced immune response is observed against an immunogen encoded by the alphavirus vector in the presence of the enhancing antibody.

Typically, the cell will be one that is permissive for alphavirus infection, although cells that are only permissive in the presence of enhancing antibody are also suitable. In preferred embodiments, the cell is an antigen-presenting cell (APC). As used herein, the term "APC" refers to cells that present antigens to T cells, including but not limited to macrophages, monocytes, dendritic cells, phagoctyic leukocytes, B lymphoctyes, and endothelial cells. Typically, APC express MHC class II antigens on the cell surface. More preferably, the cell is a dendritic cell (DC), including DC precursors such as Langerhans cells.

DC are a system of antigen-presenting cells that function to initiate several immune responses such as the sensitization of MHC-restricted T cells, the rejection of organ transplants, and the formation of T cell-dependent antibodies. DC are found in many non-lymphoid tissues but can migrate via the afferent lymph or the blood stream to the T cell-dependent areas of lymphoid organs. They are found in the skin, where they are called Langerhans cells, and are also present in the mucosa. DC are believed to function within the peripheral tissues where they acquire antigens for presentation to the immune system.

DC appear to play a crucial role in the initiation of T-cell dependent responses. These cells bind and modify antigens in a manner such that the modified antigen, when presented on the surface of the DC, can activate T-

IgG. Exemplary FcγR include FcγRI (*e.g.*, FcγRA, FcγRB and FcγRIC), FcγRII (*e.g.*, FcγRIIA, FcγRIIB and FcγRIIC), and FcγRIII (*e.g.*, FcγRIIIA or FcγRIIIB). FcγRI, present on monocytes and macrophages, is a high affinity IgG receptor. FcγRII and FcγRIII are relatively low affinity receptors and appear to only bind antibody in the form of immune complexes. FcγRII are more widely expressed by hematopoietic cells than are FcγRIII.

FcγR and FcεR couple humoral and cellular immunity by directing the interaction of antibodies with effector cells. These receptors are present on most effector cells of the immune system and mediate phagocytosis, antibody-dependent cell-mediated cytotoxicity, activation of inflammatory cells, and many of the biological sequelae associated with antibody-dependent immunity.

Cells that express FcγR include, but are not limited to, cells of hematopoietic lineage, including but not limited to, macrophages, monocytes, platelets, neutrophils, eosinophils, mast cells, natural killer cells, basophils, B cells, and DC cells. Macrophages, monocytes and DC are preferred, with DC (including Langerhans cells and other DC precursors) being more preferred. Cells expressing FcγR also include immature thymocytes and certain tumor cell lines.

In other particular embodiments, the alphavirus vector is contacted with a cell that expresses a complement receptor (*e.g.*, complement receptor 1 (CR1), CR2, CR3, and the like). The complement system includes a group of proteins in blood plasma that play an integral role in immune and allergic reactions. Activation of complement can occur via at least two pathways. The classical pathway of complement activation involves antigen-antibody complexes. Regardless of which initiation pathway is used, the end result is the formation of activated fragments of complement proteins (*e.g.*, C3a, C4a, C5a and C5b-9), which mediate several functions including leukoctye chemotaxis, activation of macrophages, vascular permeability, and cellular lysis. Exemplary cells that express complement receptors include, but are not limited to, dendritic cells, monocytes, macrophages, neutrophils, eosinophils, erythroctyes, B cells, T cells, epithelial cells, natural killer cells, mast cells,

preferably, the immunogenic peptide or protein is suitable for providing some degree of protection to a subject against a disease. The present invention may be employed to express an immunogenic peptide or protein in a subject (*e.g.*, for vaccination) or for immunotherapy (*e.g.*, to treat a subject with cancer or tumors).

An immunogenic protein or peptide, or immunogen, may be any protein or peptide suitable for protecting the subject against a disease, including but not limited to microbial, bacterial, protozoal, parasitic, and viral diseases. For example, the immunogen may be an orthomyxovirus immunogen (*e.g.*, an influenza virus immunogen, such as the influenza virus hemagglutinin (HA) surface protein or the influenza virus nucleoprotein gene, or an equine influenza virus immunogen), or a lentivirus immunogen (*e.g.*, an equine infectious anemia virus immunogen, a Simian Immunodeficiency Virus (SIV) immunogen, or a Human Immunodeficiency Virus (HIV) immunogen, such as the HIV or SIV envelope GP160 protein, the HIV or SIV matrix/capsid proteins, and the HIV or SIV gag, pol and env genes products). The immunogen may also be an arenavirus immunogen (*e.g.,* Lassa fever virus immunogen, such as the Lassa fever virus nucleocapsid protein gene and the Lassa fever envelope glycoprotein gene), a poxvirus immunogen (*e.g.*, vaccinia, such as the vaccinia L1 or L8 genes), a flavivirus immunogen (*e.g.,* a yellow fever virus immunogen or a Japanese encephalitis virus immunogen), a filovirus immunogen (*e.g.*, an Ebola virus immunogen, or a Marburg virus immunogen, such as NP and GP genes), a bunyavirus immunogen (*e.g.*, RVFV, CCHF, and SFS viruses), or a coronavirus immunogen (*e.g.*, an infectious human coronavirus immunogen, such as the human coronavirus envelope glycoprotein gene, or a porcine transmissible gastroenteritis virus immunogen, or an avian infectious bronchitis virus immunogen). The immunogen may further be a polio antigen, herpes antigen (*e.g.*, CMV, EBV, HSV antigens) mumps antigen, measles antigen, rubella antigen, diptheria toxin or other diptheria antigen, pertussis antigen, hepatitis (*e.g.*, hepatitis A or hepatitis B) antigen, or any other vaccine antigen known in the art.

symptoms that result from an absence or defect in a protein in a cell or subject. Alternatively, a "therapeutic" peptide or protein is one that otherwise confers a benefit to a subject, *e.g.*, anti-cancer effects. Therapeutic peptides and proteins include, but are not limited to, CFTR (cystic fibrosis transmembrane regulator protein), dystrophin (including the protein product of dystrophin mini-genes, *see, e.g*, Vincent *et al.*, (1993) *Nature Genetics* **5**:130), utrophin (Tinsley *et al.*, (1996) *Nature* **384**:349), clotting factors (Factor XIII, Factor IX, Factor X, *etc.)*, erythropoietin, the LDL receptor, lipoprotein lipase, ornithine transcarbamylase, β-globin, α-globin, spectrin, α-antitrypsin, adenosine deaminase, hypoxanthine guanine phosphoribosyl transferase, β-glucocerebrosidase, sphingomyelinase, lysosomal hexosaminidase, branched-chain keto acid dehydrogenase, hormones, growth factors (*e.g.*, insulin-like growth factors 1 and 2, platelet derived growth factor, epidermal growth factor, nerve growth factor, neurotrophic factor –3 and –4, brain-derived neurotrophic factor, glial derived growth factor, transforming growth factor-α and –β, and the like), cytokines (*e.g.*, α-interferon, β-interferon, γ-interferon, ω-interferon, τ-interferon, interleukin-1α, interleukin-1β, interleukin-2, interleukin-3, interleukin-4, interleukin 5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-10, interleukin-11, interleukin 12, interleukin-13, interleukin-14, interleukin-18, B cell Growth factor, CD40 Ligand, tumor necrosis factor-α, tumor necrosis factor-β, monocyte chemoattractant protein-1, granulocyte-macrophage colony stimulating factor, lymphotoxin), suicide gene products (*e.g.*, herpes simplex virus thymidine kinase, cytosine deaminase, diphtheria toxin, cytochrome P450, deoxycytidine kinase, and tumor necrosis factor), proteins conferring resistance to a drug used in cancer therapy, tumor suppressor gene products (*e.g.*, p53, Rb, Wt-1, NF1, VHL, APC, and the like), and any other peptide or protein that has a therapeutic effect in a subject in need thereof.

Further exemplary therapeutic peptides or proteins include those that may used in the treatment of a disease condition including, but not limited to, cystic fibrosis (and other diseases of the lung), hemophilia A, hemophilia B, thalassemia, anemia and other blood disorders, AIDS, Alzheimer's disease,

sites (IRES), promoters, enhancers, and the like. Those skilled in the art will appreciate that a variety of promoter/enhancer elements may be used depending on the level and tissue-specific expression desired. The promoter/enhancer may be constitutive or inducible, depending on the pattern of expression desired. The promoter/enhancer may be native or foreign and can be a natural or a synthetic sequence.

Promoters/enhancers that are native to the subject to be treated are most preferred. Also preferred are promoters/enhancers that are native to the heterologous nucleic acid sequence. The promoter/enhancer is chosen so that it will function in the target cell(s) of interest. Mammalian promoters/enhancers are also preferred.

Preferably, the heterologous nucleotide sequence is operably associated with a promoter that provides high level expression of the heterologous nucleotide sequence, *e.g.*, an alphavirus subgenomic 26S promoter (preferably, a VEE 26S promoter).

In embodiments of the invention in which the heterologous nucleic acid sequence(s) will be transcribed and then translated in the target cells, specific initiation signals are generally required for efficient translation of inserted protein coding sequences. These exogenous translational control sequences, which may include the ATG initiation codon and adjacent sequences, can be of a variety of origins, both natural and synthetic.

As a further aspect, the present invention provides a method of targeting an alphavirus vector of the invention to a particular cell type(s) (*i.e.*, directing the alphavirus vector to a particular cell or cell types) comprising contacting the cell with an alphavirus vector carrying a heterologous nucleotide sequence in the presence of an enhancing antibody directed against the alphavirus. In other words, the alphavirus vector infects the cell at a higher rate than it would in the absence of the antibody. Preferably, the cell is contacted (as this term is described hereinabove) with an alphavirus vector comprising a heterologous nucleotide sequence and an antibody that specifically binds to the alphavirus vector, whereby the heterologous nucleotide sequence is introduced into and expressed in the cell.

Immunophysiology: Cell Function and Cellular Interactions in Antibody Formation, in IMMUNOLOGY: BASIC PROCESSES 117 (Joseph A. Bellanti ed., 1985). Alternatively stated, an active immune response is mounted by the host after exposure to immunogens by infection or by vaccination. Active immunity can be contrasted with passive immunity, which is acquired through the "transfer of preformed substances (antibody, transfer factor, thymic graft, interleukin-2) from an actively immunized host to a non-immune host." *Id.*

A "protective" immune response or "protective" immunity as used herein indicates that the immune response confers some benefit to the subject in that it prevents or reduces the incidence of disease. Alternatively, a protective immune response or protective immunity may be useful in the treatment of disease, in particular cancer or tumors (*e.g.*, by causing regression of a cancer or tumor and/or by preventing metastasis and/or by preventing growth of metastatic nodules). The protective effects may be complete or partial, as long as the benefits of the treatment outweigh any disadvantages thereof.

In particular embodiments, an alphavirus vector comprising a heterologous nucleotide sequence encoding an immunogen and an antibody that specifically binds to the alphavirus vector are administered to a subject.

In other particular preferred embodiments, the alphavirus vector is contacted with the enhancing antibody that specifically binds to the alphavirus vector to form a complex. As described hereinabove, the complex may then administered to the subject. Alternatively, the antibody may be administered prior to, or subsequent to, the administration of the alphavirus vector.

As an alternative embodiment, the antibody may be provided by the subject (*e.g.*, by a prior immunization with an alphavirus vector to produce an immune response thereto), as described herein.

As a further alternative, the alphavirus vector may be administered to a cell *ex vivo* and the altered cell is administered to the subject. According to this embodiment, an alphavirus vector comprising a heterologous nucleotide sequence encoding an immunogen is contacted with a cell in the presence of an enhancing antibody that specifically binds to the alphavirus vector, as

The approach disclosed herein provides a generalized strategy for treating and preventing cancers of any origin, either tumor forming or non-tumor forming cancers. The inventive methods can be used to treat both the primary cancer or tumor and to prevent metastasis. Alternatively, the inventive methods can be advantageously employed to reduce or prevent growth of metastatic nodules (*e.g.*, following surgical removal of a primary tumor). The term "cancer" has its understood meaning in the art, for example, an uncontrolled growth of tissue that has the potential to spread to distant sites of the body (*i.e.*, metastasize). Exemplary cancers include, but are not limited to, leukemias, lymphomas, colon cancer, renal cancer, liver cancer, breast cancer, lung cancer, prostate cancer, ovarian cancer, melanoma, and the like. Preferred are methods of treating and preventing tumor-forming cancers. The term "tumor" is also understood in the art, for example, as an abnormal mass of undifferentiated cells within a multicellular organism. Tumors can be malignant or benign. Preferably, the inventive methods disclosed herein are used to prevent and treat malignant tumors.

Cancer and tumor antigens according to the present invention have been described hereinabove. By the terms "treating cancer" or "treatment of cancer", it is intended that the severity of the cancer is reduced or the cancer is at least partially eliminated. Preferably, these terms indicate that metastasis of the cancer is reduced or at least partially eliminated. It is further preferred that these terms indicate that growth of metastatic nodules (*e.g.*, after surgical removal of a primary tumor) is reduced or at least partially eliminated. By the terms "prevention of cancer" or "preventing cancer" it is intended that the inventive methods at least partially eliminate or reduce the incidence or onset of cancer. Alternatively stated, the present methods slow, control, decrease the likelihood or probability, or delay the onset of cancer in the subject.

Likewise, by the terms "treating tumors" or "treatment of tumors", it is intended that the severity of the tumor is reduced or the tumor is at least partially eliminated. Preferably, these terms are intended to mean that metastasis of the tumor is reduced or at least partially eliminated. It is also

Cytokines may be administered by any method known in the art. Exogenous cytokines may be administered to the subject, or alternatively, a nucleotide sequence encoding a cytokine may be delivered to the subject using a suitable vector, and the cytokine produced *in vivo*.

In preferred embodiments, an alphavirus vector encoding a cytokine is used to deliver the cytokine to the subject. Accordingly, the present invention further provides a method of delivering a cytokine to a cell (preferably APC, more preferably DC, as described hereinabove) comprising contacting the cell with an alphavirus vector carrying a heterologous nucleotide sequence encoding the cytokine in the presence of an antibody against the alphavirus, whereby the nucleotide sequence encoding the cytokine is introduced into and expressed by the cell. This method may be employed to enhance an immune response to an antigen, *e.g.*, a vaccine antigen or a cancer or tumor cell antigen.

In particular embodiments, an alphavirus vector encoding an antigen and a cytokine may be contacted with and introduced into a cell. Alternatively, the antigen may be delivered by one alphavirus vector, and the cytokine is introduced into the cell by means of a different alphavirus vector.

II. Alphavirus Vectors.

The present invention is practiced using alphavirus vectors, more preferably a propagation-incompetent alphavirus vector, still more preferably an alphavirus replicon vector. Alphavirus and replicon vectors are described in U.S. Patent No. 5,505,947 to Johnston et al.; U.S. Patent No. 5,792,462 to Johnston et al., U.S. Patent No. 5,814,482 to Dubensky et al., U.S. Patent No. 5,843,723 to Dubensky et al., U.S. Patent No. 5,789,245 to Dubensky et al., U.S. Patent No. 5,739,026 to Garoff et al., the disclosures of which are incorporated herein by reference in their entirety.

Alphavirus replicon vectors, and in particular VEE replicon vectors, elicit a strong host response to immunogen. While not wishing to be held to any particular theory of the invention, it appears that alphavirus replicon vectors induce a more balanced and comprehensive immune response (*i.e.*, cellular and

Arbovirus No. 86, Ockelbo virus, Girdwood S.A. virus, Aura virus, Whataroa virus, Babanki virus, and Kyzylagach virus.

Preferred are alphaviruses including attenuating mutations. The phrases "attenuating mutation" and "attenuating amino acid," as used herein, mean a nucleotide sequence containing a mutation, or an amino acid encoded by a nucleotide sequence containing a mutation, which mutation results in a decreased probability of causing disease in its host (*i.e.*, a loss of virulence), in accordance with standard terminology in the art. *See, e.g.,* B. Davis et al., MICROBIOLOGY 132 (3d ed. 1980). The phrase "attenuating mutation" excludes mutations or combinations of mutations which would be lethal to the virus.

Appropriate attenuating mutations will be dependent upon the alphavirus used. Suitable attenuating mutations within the alphavirus genome will be known to those skilled in the art. Exemplary attenuating mutations include, but are not limited to, those described in United States Patent No. 5,505,947 to Johnston et al., U.S. Patent No. 5,185,440 to Johnston et al., U.S. Patent No. 5,643,576 to Davis et al., U.S. Patent No. 5,792,462 to Johnston et al., and U.S. Patent No. 5,639,650 to Johnston et al., the disclosures of which are incorporated herein in their entirety by reference.

When the alphavirus capsid is from VEE, suitable attenuating mutations may be selected from the group consisting of codons at E2 amino acid position 76 which specify an attenuating amino acid, preferably lysine, arginine, or histidine as E2 amino acid 76; codons at E2 amino acid position 120 which specify an attenuating amino acid, preferably lysine as E2 amino acid 120; codons at E2 amino acid position 209 which specify an attenuating amino acid, preferably lysine, arginine or histidine as E2 amino acid 209; codons at E1 amino acid 272 which specify an attenuating amino acid, preferably threonine or serine as E1 amino acid 272; codons at E1 amino acid 81 which specify an attenuating amino acid, preferably isoleucine or leucine as E1 amino acid 81; codons at E1 amino acid 253 which specify an attenuating amino acid, preferably serine or threonine as E1 amino acid 253; or the deletion of E3 amino acids 56-69, or a combination of the deletion of E3 amino acids 56-59 together

Also preferred are alphavirus vectors in which there is a mutation in the capsid protease that reduces, preferably ablates, the autoprotease activity of the capsid and results, therefore, in non-viable virus. Capsid mutations that reduce or ablate the autoprotease activity of the alphavirus capsid are known in the art, *see e.g.*, WO 96/37616 to Johnston et al., the disclosure of which is incorporated herein in its entirety. In particular embodiments, the alphavirus vector comprises a VEE virion or VEE capsid proteins in which the capsid protease is ablated, *e.g.*, by introducing an amino acid substitution at VEE capsid position 152, 174, or 226.

Mutations may be introduced into the alphavirus vector by any method known in the art. For example, mutations may be introduced into the alphavirus RNA by performing site-directed mutagenesis on the cDNA which encodes the RNA, in accordance with known procedures. *See*, Kunkel, *Proc. Natl. Acad. Sci. USA* **82**, 488 (1985), the disclosure of which is incorporated herein by reference in its entirety. Alternatively, mutations may be introduced into the RNA by replacement of homologous restriction fragments in the cDNA which encodes for the RNA, in accordance with known procedures.

The alphavirus vector may be a chimeric alphavirus, as that term is understood in the art. For example, the alphavirus virion (*i.e.*, the structural proteins) may be from one alphavirus (preferably, VEE) and the nucleic acid packaged within the capsid be from another alphavirus, or any other virus. Alternatively, the alphavirus virus may be assembled from structural proteins derived from more than one alphavirus.

According to particular embodiments, it is desirable to employ an alphavirus vector that encodes two or more (*e.g.*, two, three, four, five, *etc.*) heterologous nucleic acid sequences, preferably each encoding an antigen according to the present invention. Each heterologous nucleic acid sequence will typically be operably associated with a promoter. Alternatively, an internal ribosome entry site (IRES) sequence(s) can be placed downstream of a promoter and upstream of the heterologous nucleic acid sequence(s). The heterologous nucleic acid sequences can be associated with a constitutive or

cases, the function of these cloned genes is known. In general, the above disease states fall into two classes: deficiency states, usually of enzymes, which are generally inherited in a recessive manner, and unbalanced states, at least sometimes involving regulatory or structural proteins, which are inherited in a dominant manner. For deficiency state diseases, gene transfer could be used to bring a normal gene into affected tissues for replacement therapy, as well as to create animal models for the disease using antisense mutations. For unbalanced disease states, gene transfer could be used to create a disease state in a model system, which could then be used in efforts to counteract the disease state. Thus the methods of the present invention permit the treatment of genetic diseases. As used herein, a disease state is treated by partially or wholly remedying the deficiency or imbalance that causes the disease or makes it more severe. The use of site-specific integration of nucleic sequences to cause mutations or to correct defects is also possible.

The instant invention may also be employed to provide an antisense nucleic acid to a cell *in vitro* or *in vivo*. Expression of the antisense nucleic acid in the target cell diminishes expression of a particular protein by the cell. Accordingly, antisense nucleic acids may be administered to decrease expression of a particular protein in a subject in need thereof. Antisense nucleic acids may also be administered to cells *in vitro* to regulate cell physiology, *e.g.*, to optimize cell or tissue culture systems. The present invention is also useful to deliver other non-translated RNAs, *e.g.*, ribozymes (*e.g.*, as described in U.S. Patent No. 5,877,022), RNAs that effect spliceosome-mediated *trans*-splicing (Puttaraju *et al.*, (1999) *Nature Biotech.* **17**:246), or "guide" RNAs (*see, e.g.*, Gorman *et al.*, (1998) *Proc. Nat. Acad. Sci. USA* **95**:4929; U.S. Patent No. 5,869,248 to Yuan *et al.*) to a target cell.

Finally, the instant invention finds further use in diagnostic and screening methods, whereby a gene of interest is transiently or stably expressed in a cell culture system, or alternatively, a transgenic animal model.

By "pharmaceutically acceptable" it is meant a material that is not biologically or otherwise undesirable, *e.g.*, the material may be administered to a subject without causing any undesirable biological effects. Thus, such a pharmaceutical composition may be used, for example, in transfection of a cell *ex vivo* or in administering the alphavirus/antibody compositions or cells directly to a subject.

The alphavirus vectors of the invention may be administered to elicit an immunogenic response (*e.g.*, as an immunogenic composition or as a vaccine or for immunotherapy). Typically, immunological compositions of the present invention comprise an immunogenic amount of infectious virus particles as disclosed herein in combination with a pharmaceutically-acceptable carrier. An "immunogenic amount" is an amount of the infectious virus particles that is sufficient to evoke an immune response in the subject to which the pharmaceutical formulation is administered. Typically, an amount of about 10^3 to about 10^{15} virus particles, preferably about 10^4 to about 10^{10}, and more preferably about 10^4 to 10^6 virus particles per dose is suitable, depending upon the age and species of the subject being treated, and the immunogen against which the immune response is desired. Subjects and immunogens are as described above.

Typically, the present invention permits lower dosages of the alphavirus vector and/or the expressed antigen to be administered to achieve an effective immune response.

The terms "vaccination" or "immunization" are well-understood in the art. For example, the terms vaccination or immunization can be understood to be a process that increases an subject's immune reaction to antigen and therefore to resist or overcome infection. In the case of the present invention, vaccination or immunization may also increase the organism's immune response and resistance to invasion by cancer or tumor cells.

Any suitable vaccine and method of producing an immune response (*i.e.*, immunization) known in the art may be employed in carrying out the present invention, as long as an active immune response (preferably, a protective immune response) against the antigen is elicited.

A "therapeutically-effective" amount as used herein is an amount that is sufficient to alleviate (*e.g.*, mitigate, decrease, reduce) at least one of the symptoms associated with a disease state. Alternatively stated, a "therapeutically-effective" amount is an amount that is sufficient to provide some improvement in the condition of the subject.

A further aspect of the invention is a method of treating subjects *in vivo* with the inventive alphavirus particles. Administration of the alphavirus particles of the present invention to a human subject or an animal in need thereof can be by any means known in the art for administering virus vectors.

Dosages of the inventive alphavirus particles will depend upon the mode of administration, the disease or condition to be treated, the individual subject's condition, the particular virus vector, and the gene to be delivered, and can be determined in a routine manner. Exemplary doses for achieving therapeutic effects are virus titers of at least about 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^3, 10^{14}, 10^{15} transducing units or more, preferably about $10^8 - 10^{13}$ transducing units.

Dosages of the antibody directed against the alphavirus vector for *in vitro*, *in vivo*, or *ex vivo* applications may be routinely determined. The antibody is preferably present in an "enhancing amount" that is sufficient to enhance infectivity of the alphavirus vector, more preferably the immune response to the heterologous antigen encoded by the vector, but insufficient to neutralize the alphavirus vector. It is also preferred that the antibody is present in an "enhancing" amount that is sufficient to target or direct the alphavirus particle to a particular cell type(s). Those skilled in the art will appreciate that the concentration of the enhancing antibody to be used will depend on several factors, including: the nature of the antibody, the alphavirus, and the antigen, the condition of the subject, and the desired result.

Exemplary modes of administration for alphavirus vectors, antibodies, and cells according to the present invention include oral, rectal, transmucosal, topical, transdermal, inhalation, parenteral (*e.g.*, intravenous, subcutaneous, intradermal, intramuscular, and intraarticular) administration, and the like, as

Example 1

Materials and Methods

Virus: VEE replicon particles (VRP) expressing either influenza virus hemagglutinin (HA-VRP-3000 and HA-VRP-3014) or the green fluorescent protein (GFP-VRP-3014 and GFP-VRP-3014) were prepared as previously described (MacDonald and Johnston, 2000 *J. Virology* **74**:914). Briefly, RNA transcripts from replicon cDNA plasmids encoding the appropriate heterologous gene were co-electroporated with RNA transcripts from two helper constructs encoding either VEE capsid or VEE glycoprotein genes into baby hamster kidney (BHK) cells. VRP were harvested directly from the culture supernates 24 hr following electroporation and titered on BHK cells. For these studies, VRP were produced using a glycoprotein helper that contained the V3014 attenuating mutations in E1 272 (ala→thr) and E2 209 (glu→lys) (Davis et al., (1991) *Virology* **183**:20).

Mice and Cells: Seven- to eight-week-old female CD1 out bred mice (Charles River Laboratory) were inoculated subcutaneously (sc) in the left rear foot pad with $5x10^5$ infectious units (IU) of VEE viral replicon particles (VRP) unless otherwise specified. Mice were perfused with 4% paraformaldehyde (PFA) in PBS 24 hr post-inoculation (pi) and the draining popliteal lymph nodes were removed to PFA. Fixed frozen sections were analyzed by fluorescent microscopy for cells expressing GFP.

Bone marrow (BM) cells were isolated from the femurs of C57BL6 mice. Cells were grown as previously described. Briefly, marrow was flushed from femurs and tibia and resuspended in PBS. Cells were washed and re-suspended in RPMI1640 supplemented with 10% FBS, L-glutamine, nonessential amino acids, sodium pyruvate, 50μM β-2-mercaptoethanol, 25mM HEPES. Cultures were supplemented with 0.1 ng/ml GM-CSF alone or with either 5% conditioned culture medium from the epidermal fibroblast cell line, NS46 (Xu et al., (1995) *J. Immunol.* **154**:2697) or 1ng/ml IL-4 and grown on standard tissue culture plastic. The loosely adherent cells were removed to passage or to test for ADE by vigorous pipetting in the presence of 0.3mM

Example 2

In vitro Infection of Bone Marrow-Derived Dendritic Cells by VEE

An *in vitro* cell culture system for VEE infection was established and optimized following protocols used to grow dendritic cells from bone marrow. Cells grown by standard protocols for growing dendritic cells from bone marrow, i.e. in either GM-CSF alone (BM) or GM-CSF and IL-4 (BM-IL-4) resulted in cells which were poorly permissive for VEE with only 0.1% of cells infected. When bone marrow cells were grown under the same conditions to establish Langerhans cell lines from skin (BM-NS; GM-CSF and conditioned media from an epidermal fibroblast cell line, NS47; Xu et al., (1995) *J. Immunol.* **154**:2697) resulted in a ten to twenty-fold increase in specific infectivity by VEE. This is consistent with the previous observation that VEE preferentially infects Langerhans cells *in vivo* when given by a subcutaneous route of inoculation (MacDonald and Johnston, 2000 *J. Virology* **74**:914). Analysis of cell surface markers demonstrated that cells grown under these conditions were negative for the T lymphocyte marker CD3 but shared dendritic cell (DEC 205 and CD11c) and macrophage (CD11b and F4/80) markers (**Fig. 1**). These cells were also positive for Fcγ receptor II/III and complement receptor I. Interestingly, while these cells were positive for MHC class I, they were negative for MHC class II, suggesting that these cells represent an early stage in dendritic cell differentiation.

Example 3

Antibody to VEE Enhances Infection of BM-NS *in vitro*

Antibodies to Sindbis, a closely related alphavirus, have been shown to enhance infectivity of this virus on BHK cells independently of Fc receptors or complement (Flynn et al., (1988) *Virology* **166**:82). Therefore, the potential enhancing activity of a rabbit anti-VEE antiserum previously shown to bind VEE infected cells (MacDonald and Johnston, 2000 *J. Virology* **74**:914) was determined on BHK cells. In contrast to Sindbis, pre-treatment of VRP with VEE-specific antibodies diluted up to 1/6400 completely neutralized infection (**Fig. 6**). However, the same treatment of VRP with antibodies enhanced

Langerhans cell lines, XS106 demonstrated ADE. These results indicate that FcγRII/III mediated uptake of the virus is not sufficient for productive viral infection, suggesting a block downstream of ADE mediated viral entry. In contrast, antibody to VEE neutralized infection on the VEE-permissive BHK cell line, cells that are negative for FcγR and complement receptors, illustrating the neutralizing capacity of these antibodies.

Example 6

Antibody to VEE Alters Cell Targeting *in vivo*

To determine if antibody to VEE could affect cell targeting *in vivo*, mice were inoculated with VRP alone or VRP pre-incubated either in control serum or anti-VEE serum and the draining lymph nodes were examined for GFP-positive cells (**Fig. 7A-7D**). A single dose of VRP packaged in the vaccine glycoprotein coat (3014) and at a dose used in vaccine protocols ($5x10^5$infectious units; IU) resulted in a limited number of predominantly small, round cells located mostly in the medulla, with occasional GFP-positive cells with dendritic cell morphology associated with B cell follicles (**Fig. 7A**). Pre-incubation of the VRP with a polyclonal VEE-specific antibody resulted in the appearance of a significant number of GFP-positive cells with Langerhans cell-like morphology just under the capsule, similar to what is seen with VRP packaged in wild type glycoproteins (**Fig. 7C**; MacDonald and Johnston, 2000 *J. Virology* **74**:914). These results demonstrate that ADE can affect cell targeting *in vivo*.

Vaccination of mice with VRP historically induces levels of VEE-specific antibodies that are undetectable by ELISA assays. Likewise prior immunity to VRP delivered heterologous antigens does not interfere with the generation of an immune response to subsequent VRP vaccines. These results suggest either that there is no antibody generated to the VRP themselves or that this antibody does not effectively neutralize the inoculated VRP. To test this, the effect of two sequential inoculations with VRP on cell targeting was determined. Mice that had been inoculated three weeks previously with VRP-3014 expressing influenza HA (HA-VRP-3014) or naïve

That Which is Claimed is:

1. A method of introducing and expressing a nucleotide sequence in a cell, comprising contacting a cell with (a) an alphavirus vector comprising a heterologous nucleotide sequence, and (b) an antibody that specifically binds to the alphavirus vector,

whereby the heterologous nucleotide sequence is introduced into and expressed in the cell.

2. The method of Claim 1, wherein the cell is an antigen-presenting cell.

3. The method of Claim 1, wherein the cell is selected from the group consisting of macrophages, monocytes, dendritic cells, phagoctyic leukocytes, B lymphoctyes, and endothelial cells.

4. The method of Claim 3, wherein the cell is a dendritic cell.

5. The method of Claim 4, wherein the cell is a Langerhans cell.

6. The method of Claim 1, wherein the cell expresses a Fcγ receptor.

7. The method of Claim 6, wherein the cell expresses a FcγII/III receptor.

8. The method of Claim 1, wherein the cell expresses a complement receptor.

9. The method of Claim 1, wherein the structural proteins of the alphavirus vector are VEE structural proteins.

acid, a ribozyme, an RNA that effects spliceosome-mediated *trans*-splicing, and a guide RNA.

21. The method of Claim 1, wherein the antibody is a polyclonal antibody.

22. The method of Claim 1, wherein the antibody is a monoclonal antibody.

23. The method of Claim 1, wherein the antibody binds to the alphavirus vector to form a complex between the alphavirus vector and the antibody.

24. The method of Claim 23, wherein the complex between the alphavirus vector and the antibody binds to the cell.

25. The method of Claim 1, wherein the cell is contacted with the alphavirus vector and the antibody *in vitro*.

26. The method of Claim 1, wherein the cell is contacted with the alphavirus vector and the antibody *in vivo*.

27. A method of administering a nucleotide sequence to a subject, comprising:

(a) administering an alphavirus vector comprising a heterologous nucleotide sequence to a subject; and

(b) administering an antibody that specifically binds to the alphavirus vector to the subject;

whereby the heterologous nucleotide sequence is introduced into and expressed in the subject.

(b) administering the cell to a subject,

whereby the heterologous nucleotide sequence is expressed in the subject.

36. A method of enhancing the introduction and expression of a nucleotide sequence in a cell, comprising contacting a cell with (a) an alphavirus vector comprising a heterologous nucleotide sequence, and (b) an antibody that specifically binds to the alphavirus vector,

whereby introduction and expression of the heterologous nucleotide sequence in the cell is enhanced in the presence of the antibody as compared with the level observed in the absence of the antibody.

37. A method of producing an immune response in a subject, comprising:

(a) administering an alphavirus vector comprising a heterologous nucleotide sequence encoding an immunogen to a subject; and

(b) administering an antibody that specifically binds to the alphavirus vector to the subject,

whereby an immune response is produced against the immunogen in the subject.

38. The method of Claim 37, wherein the immunogen is a cancer antigen or a tumor antigen.

39. The method of Claim 38, further comprising the step of administering a cytokine to the subject.

40. The method of Claim 39, wherein the alphavirus vector comprises the heterologous nucleotide sequence encoding the immunogen and a second heterologous nucleotide sequence encoding the cytokine.

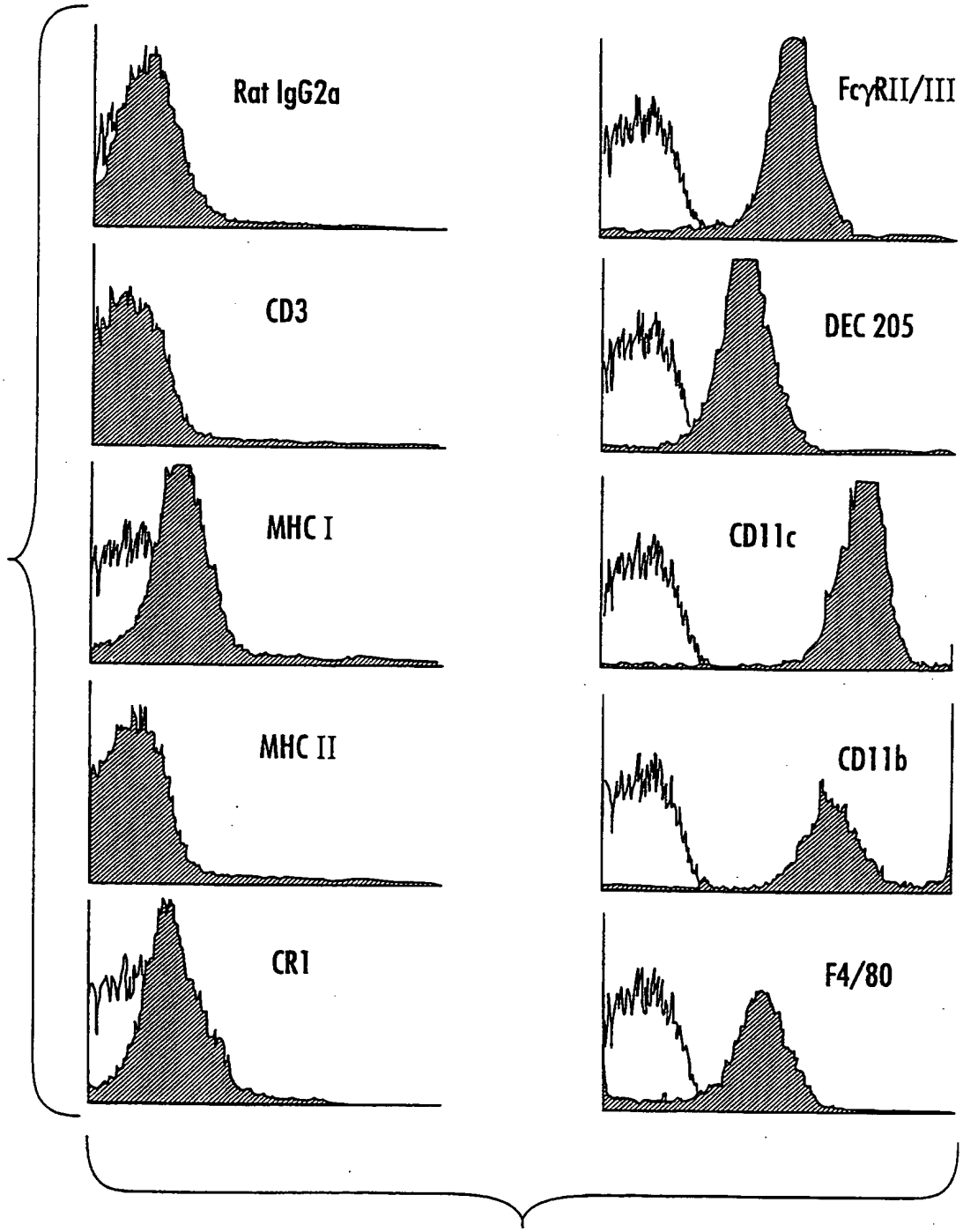

Rat IgG2a
FcγRII/III
CD3
DEC 205
MHC I
CD11c
MHC II
CD11b
CR1
F4/80

FIG. 1.

FIG. 2A.

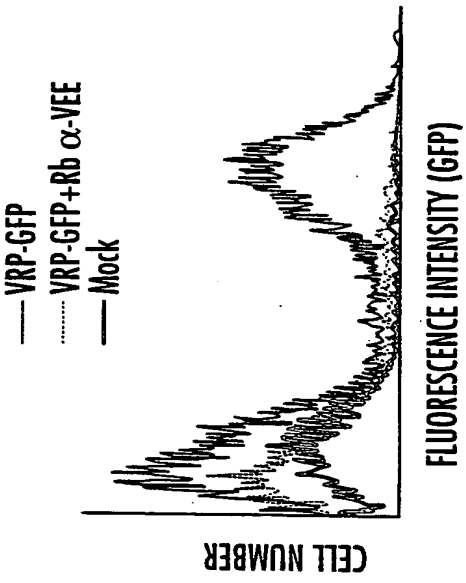
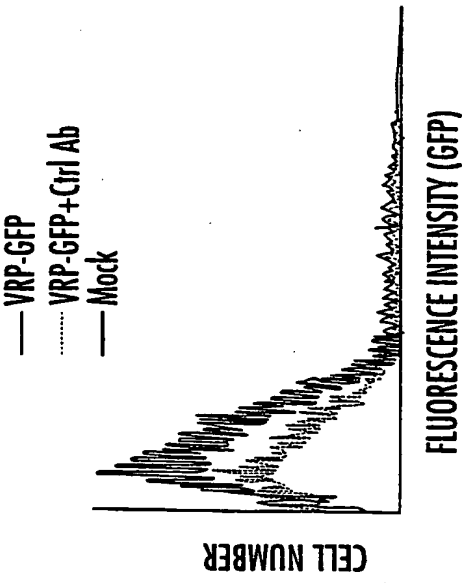

VRP-GFP
VRP-GFP+Rb α-VEE
Mock
CELL NUMBER
FLUORESCENCE INTENSITY (GFP)

FIG. 2b.


VRP-GFP
VRP-GFP+Ctrl Ab
Mock
CELL NUMBER
FLUORESCENCE INTENSITY (GFP)

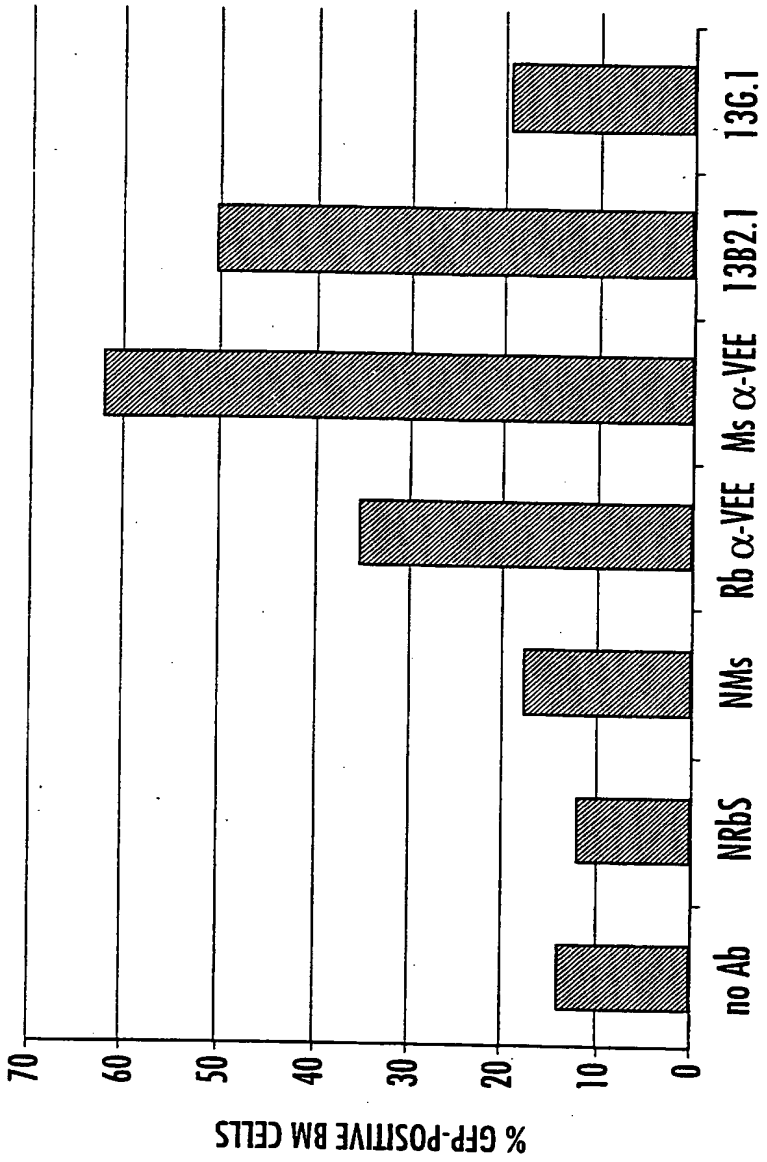

% GFP-POSITIVE BM CELLS
0
10
20
30
40
50
60
70
no Ab
NRbS
NMs
Rb α-VEE
Ms α-VEE
13B2.1
13G.1

FIG. 3.

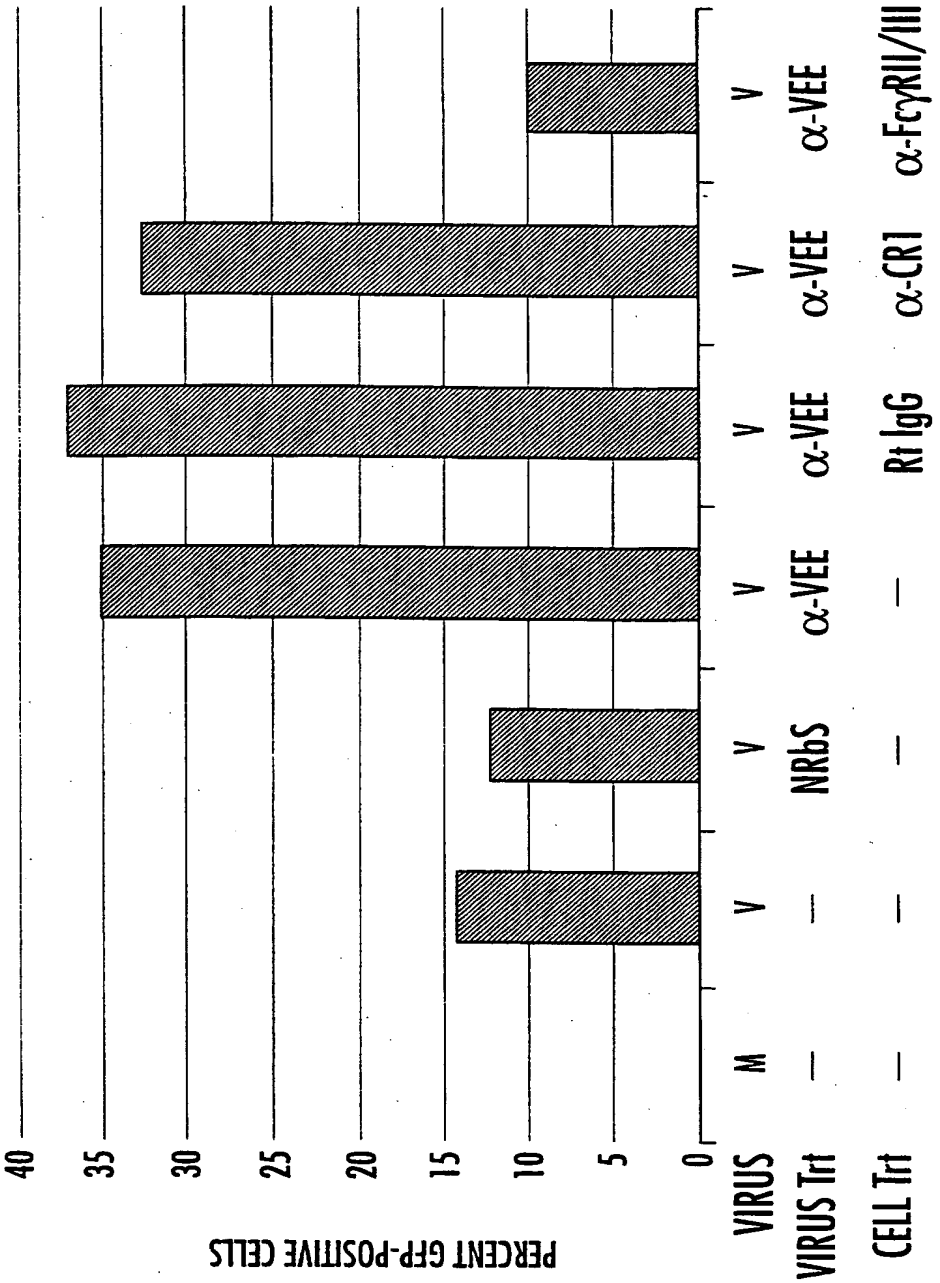

PERCENT GFP-POSITIVE CELLS
0
5
10
15
20
25
30
35
40
VIRUS
VIRUS Trt
CELL Trt
M
V
V
V
V
V
—
—
—
—
—
—
NRbS
α-VEE
α-VEE
α-VEE
α-VEE
Rt IgG
—
α-CR1
α-VEE
α-FcγRII/III

FIG. 4.

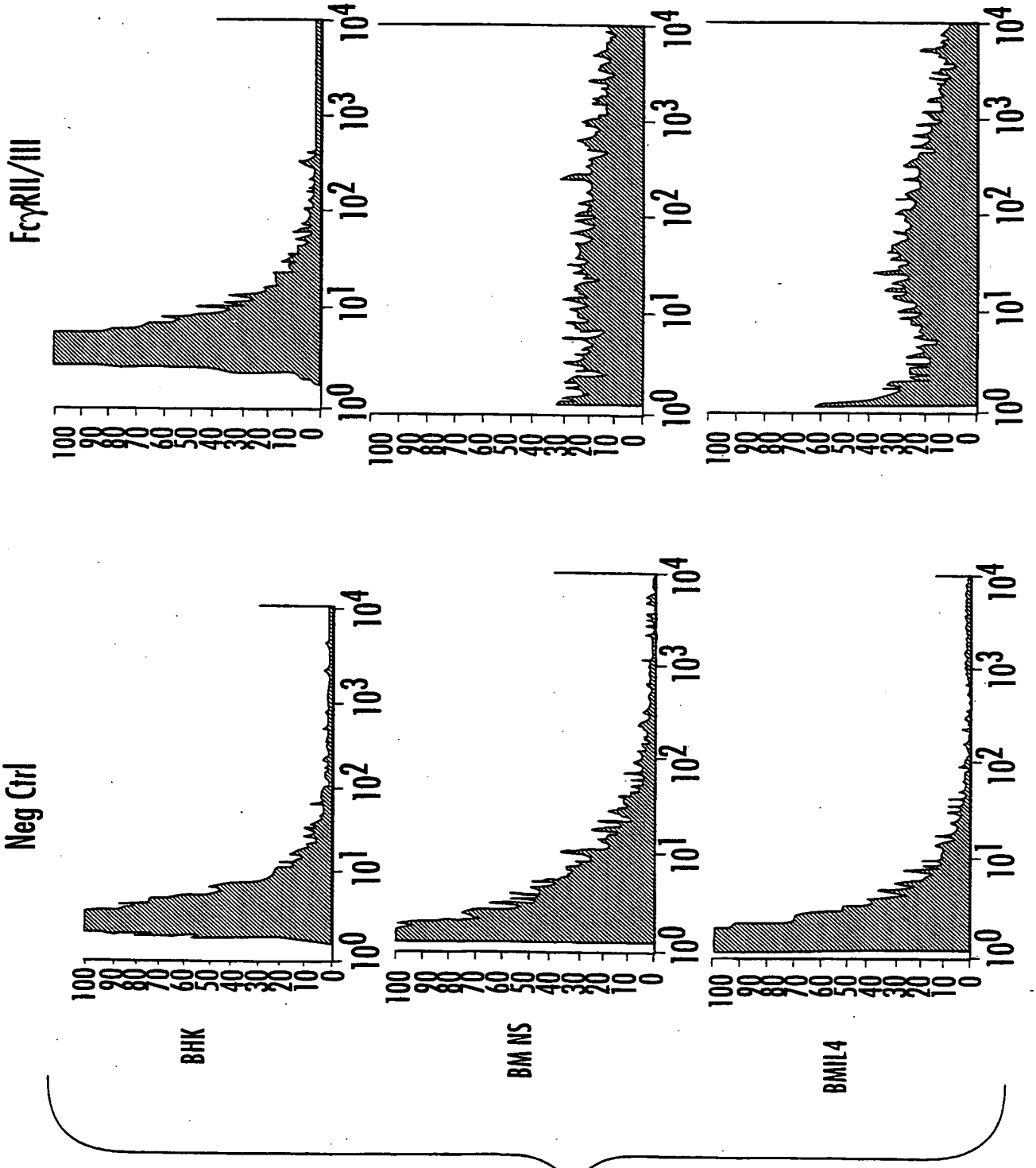


FIG. 5.

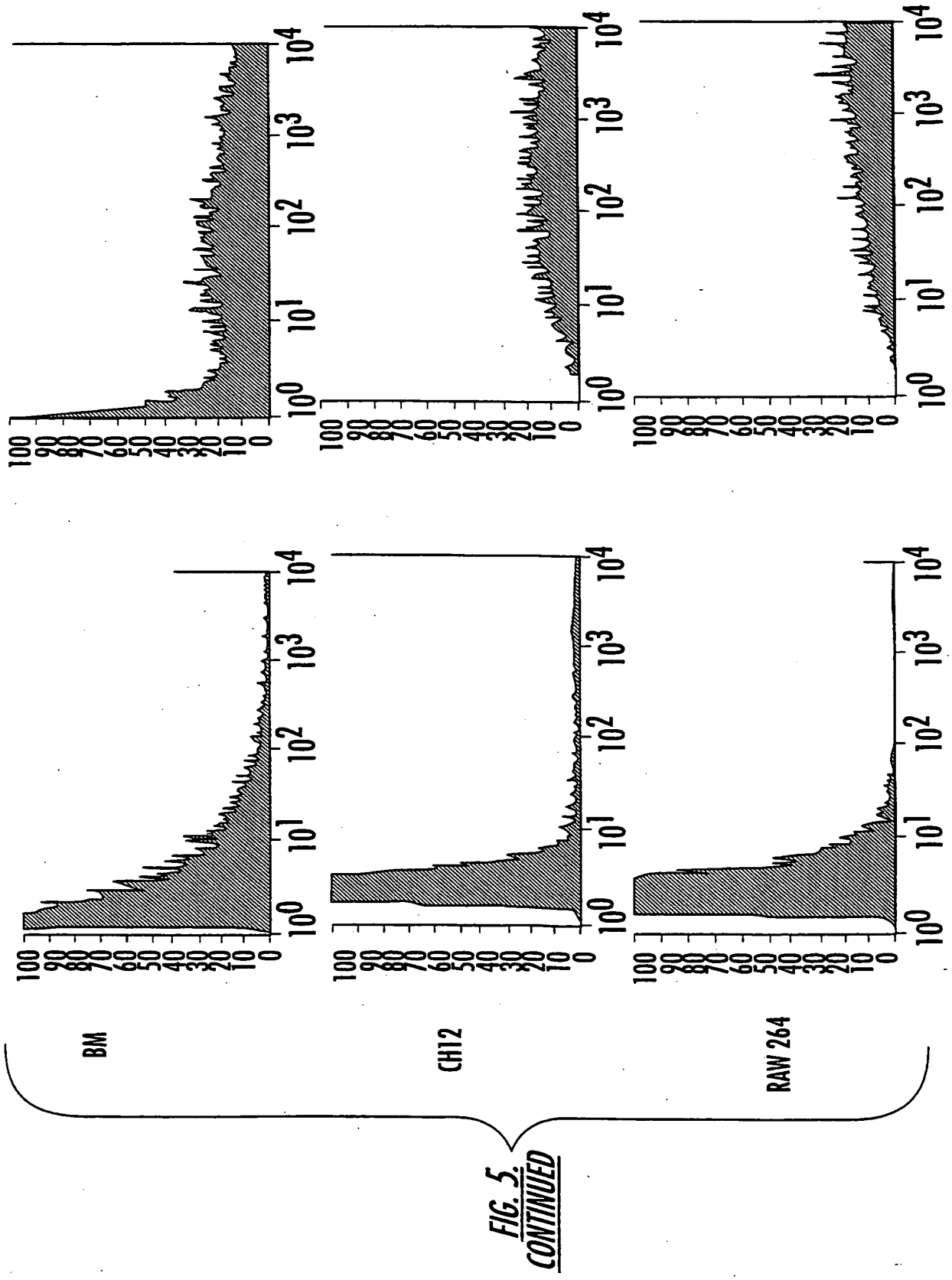
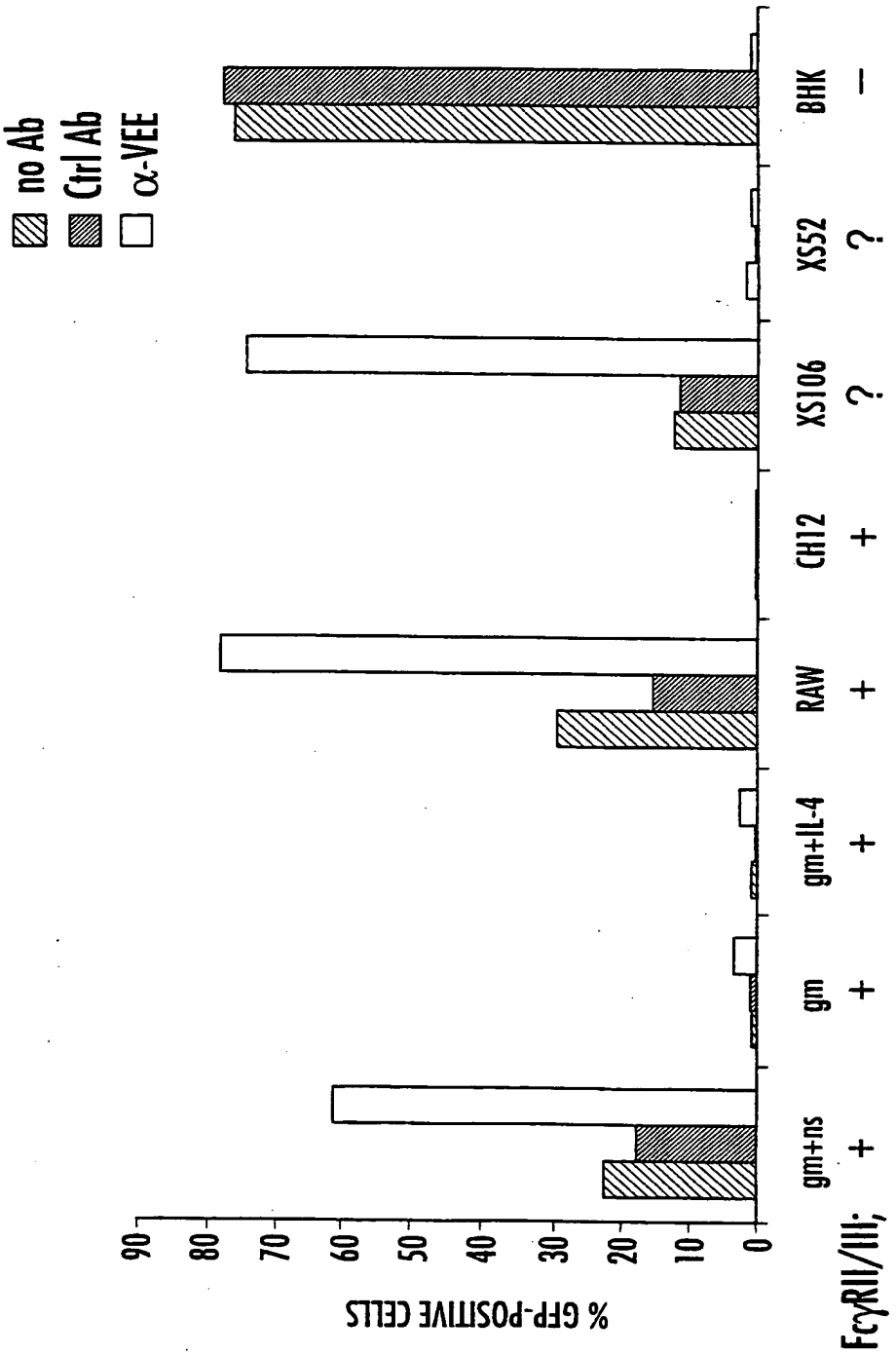


FIG. 5. CONTINUED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% GFP-POSITIVE CELLS
0
10
20
30
40
50
60
70
80
90
FcγRII/III;
gm+ns
gm
gm+IL-4
RAW
CH12
XS106
XS52
BHK
+
+
+
+
+
?
?
—

FIG. 6.

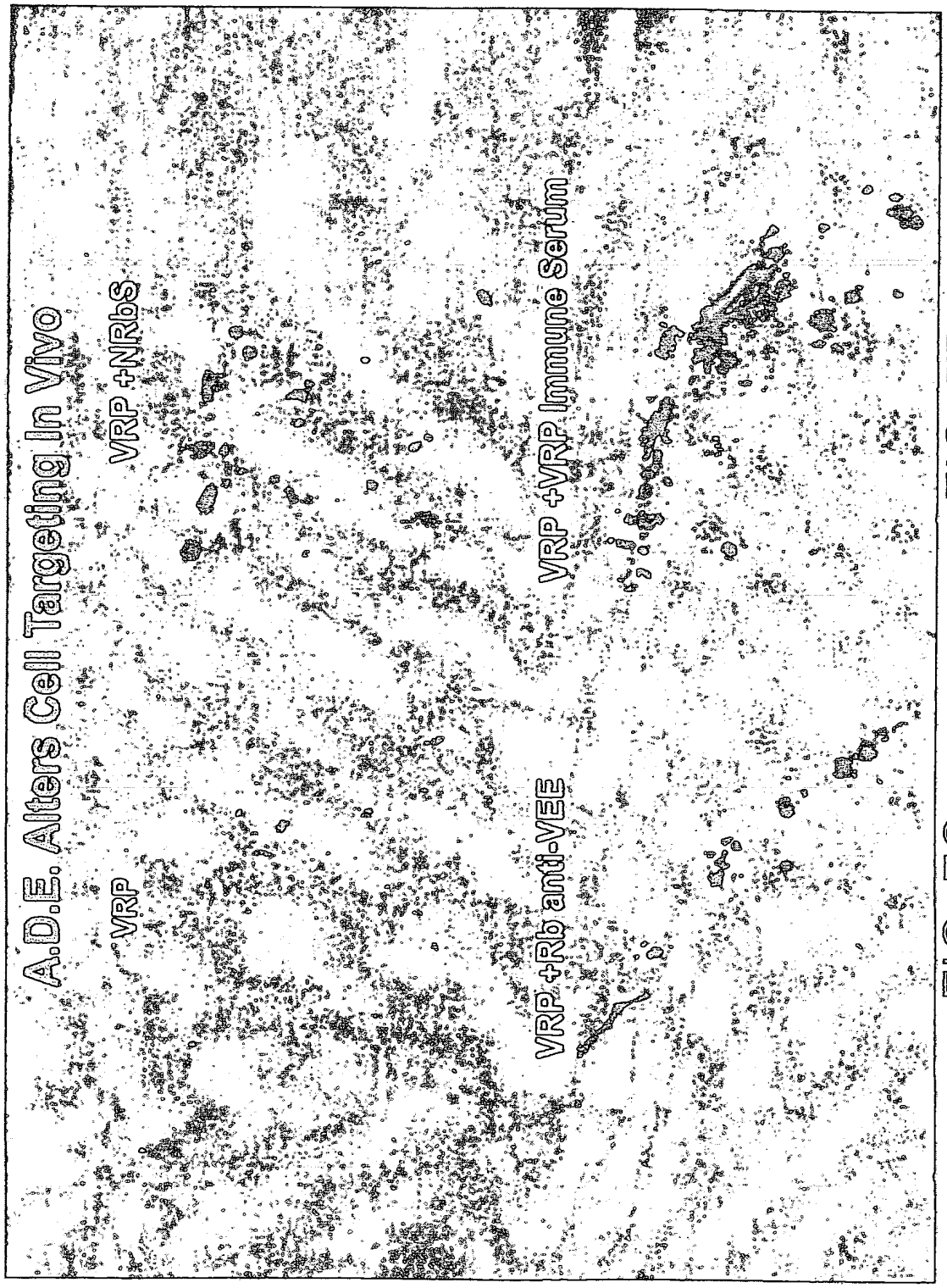

FIG. 7A.

FIG. 7B.

FIG. 7C.

FIG. 7D.

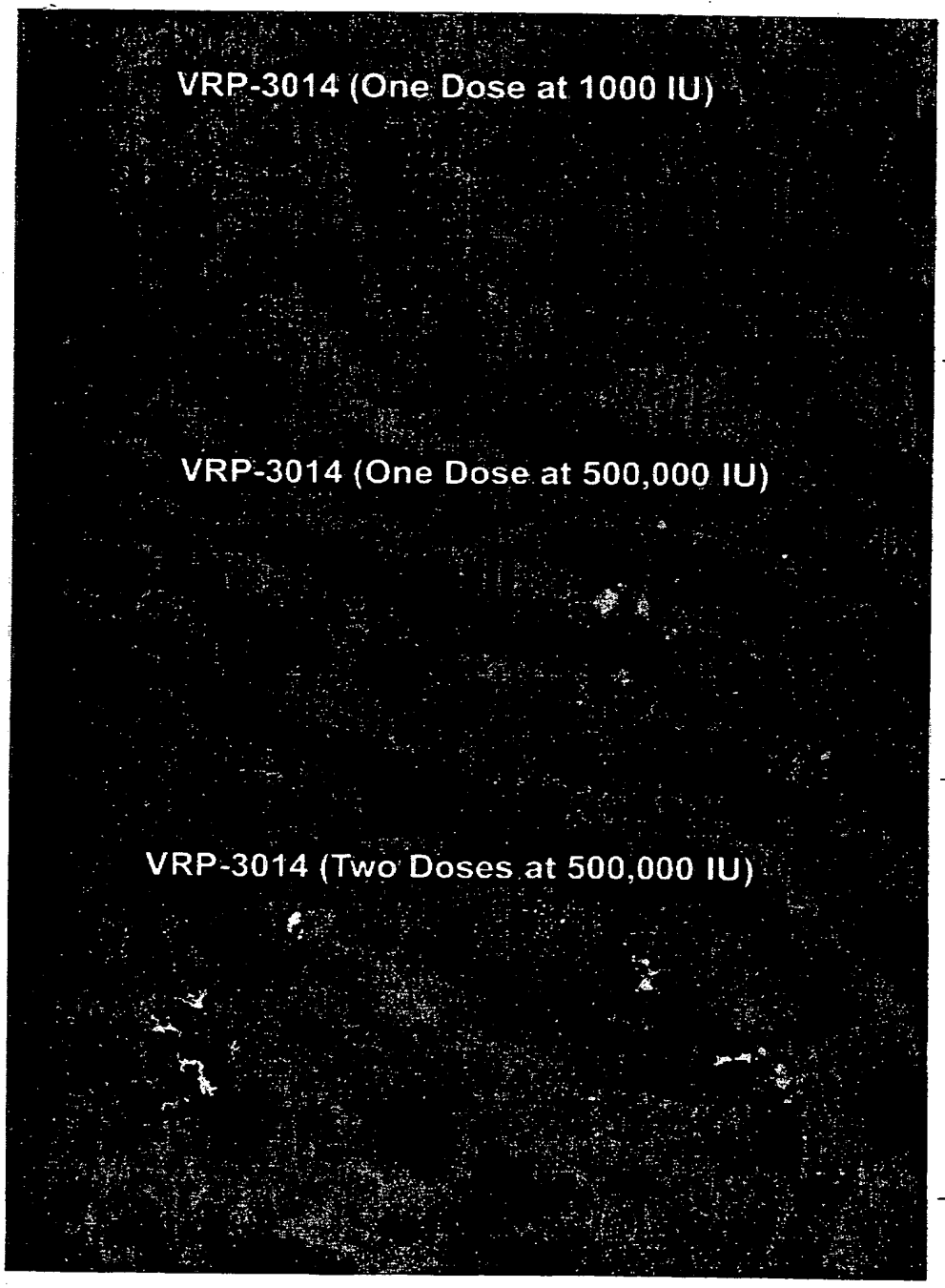

FIG. 8A.

FIG. 8B.

FIG. 8C.

INTERNATIONAL SEARCH REPORT

Internation. pplication No
PCT/US 00/23845

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/86 C12N15/87 A61K39/00 //C12N15/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 659 885 A (AKZO NOBEL NV) 28 June 1995 (1995-06-28) abstract page 2, line 10 - line 21 claims 1,8	1-45
Y	WO 95 32733 A (UNIV NORTH CAROLINA) 7 December 1995 (1995-12-07) the whole document	1-45
A	LINN M.L. ET AL.: "Antibody-dependent enhancement and persistence in macrophages of an arbovirus associated with artritis." J. GEN. VIROL., vol. 77, 1996, pages 407-411, XP002157461 the whole document	1-45

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 January 2001	05/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Galli, I

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internation pplication No
PCT/US 00/23845

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0659885 A	28-06-1995	JP 7250683 A	03-10-1995
		US 5789388 A	04-08-1998
WO 9532733 A	07-12-1995	US 5505947 A	09-04-1996
		AU 709795 B	09-09-1999
		AU 2692395 A	21-12-1995
		CA 2193210 A	07-12-1995
		EP 0762894 A	19-03-1997
		JP 10501405 T	10-02-1998
		US 5643576 A	01-07-1997